Multiple terminal uridylyltransferases of trypanosomes

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Abstract The transferase activities that add uridylyl residues to RNA have been reported in several unicellular and metazoan organisms. Thus far, the two terminal uridylyltransferases (TUTases) involved in uridine insertion/deletion mRNA editing in mitochondria of trypanosomes were the only known enzymes with confirmed UTP specificity. Here, we demonstrate that protein sequences of editing TUTases may be used to predict novel UTP-specific enzymes by data mining. The highest-scoring open reading frame from *Trypanosoma brucei* was expressed and recombinant protein purified. This enzyme catalyzes a processive UMP incorporation and is not localized to the mitochondria suggesting a non-editing biological function.

Keywords: Trypanosoma brucei; Terminal uridylyltransferases; Leishmania; RNA editing; C_2H_2 zinc finger

1. Introduction

Terminal uridylyltransferases (TUTases) add UMP residues to the 3′ hydroxyl group of RNA in a template-independent polymerization reaction. These activities have been described in mammalian cells [1–3], plants [4] and trypanosomatids [5–7]. Previous work on protein complexes involved in uridine insertion/deletion RNA editing in mitochondria of trypanosomes identified two TUTases [8]. RNA Editing TUTase 1 or RET1 [7] was implicated in the addition of the non-encoded 3′ oligo(U) tail to guide RNAs [9]. RNA Editing TUTase 2 or RET2 [10,11] was shown to be responsible for U-insertion into the mRNA during the editing cycle [9]. These proteins belong to the superfamily of nucleotidyltransferases typified by DNA polymerase β (Pol β) [12] but differ substantially in polypeptide size, RNA substrate specificity, processivity and quaternary structure. RET1 from *Leishmania tarentolae* is a tetramer of 121 kDa subunits and RET2 is a 57 kDa integral component of the core editing L-complex [7,9,11]. Although both TUTases utilize UTP as the preferred nucleotide, RET1 adds hundreds of uridines to an RNA primer in vitro, whereas RET2 transfers mainly one residue. Nevertheless, a high degree of similarity was observed between catalytic domains of these two enzymes [10].

To date, the RNA editing TUTases, RET1 and RET2, represent the only UTP-specific 3′ RNA transferases with identified genes and established functions. Despite of the apparently wide occurrence of TUTase activities in eukaryotic cells, little information is available on their possible functions beyond RNA editing. Also, the general conservation of catalytic domains among homopolynucleotide transferases, such as poly(A) polymerases and TUTases [13], and the limited number of divergent TUTase protein sequences make elucidation of nucleotide-specific recognition motifs a challenging task. In this work we inquired whether novel UTP-specific nucleotidyltransferases can be discovered by data mining with the RET1 and RET2 sequences.

2. Materials and methods

2.1. Cell cultures and protein isolation

The predicted open reading frame for TbTUT3 was amplified from *Trypanosoma brucei* genomic DNA with Pfu DNA polymerase, sequenced and inserted into previously described vector that provides a C-terminal TAP affinity tag [10]. *L. tarentolae* cells (UC) strain were transfected by electroporation and neomycin-resistant clones were selected on BHI/agar plates with 200 μ/ml of Geneticin (Invitrogen). The tandem affinity purification has been performed as described [9]. Recombinant TbRET1 was isolated as in [7].

2.2. Cell fractionation and Western blotting analysis

L. tarentolae cells were washed in PBS, re-suspended in a hypotonic buffer (10 mM Tris–HCl, 1 mM EDTA) at 10^9/ml and lysed by passing through a 26 gauge needle under pressure (6 bar). The extract was immediately supplemented with sucrose to 0.25 M, membrane fraction pelleted by centrifugation at $10\,000 \times g$ for 10 min and mitochondria further purified as described [14]. Mitochondrial fraction was re-suspended in PBS at 20 mg/ml of total protein and lysed with 0.5% NP40 for 30 min on ice. Following the 3×15 s sonication using Branson Sonifier 150 with 20k kHz frequency, the extract was clarified by centrifugation for 30 min at $18\,000 \times g$. The cytoplasmic fraction was subjected to additional centrifugation at $200\,000 \times$ g for 30 min to obtain an S100 extract. The soluble peroxidase/antiperoxidase complex (PAP reagent, Sigma) was used to detect TAP fusion protein according to manufacturer's instructions. Rabbit polyclonal antibodies were raised against recombinant proteins purified from *E. coli*. Western blot analysis was performed by standard protocols with Super Signal chemiluminescent substrate (Pierce).

2.3. Nucleotide incorporation assays

Nucleotide triphosphate incorporation assay with TbRET1 was performed in 10 μl reaction containing 20 mM Tris–HCl, pH 8.0, 10 mM KCl, 10 mM $MgCl_2$, 1 mM DTT and 10 nM of 5′-labeled 12[U] RNA oligonucleotide [7]. For the TbTUT3 assay, concentrations of $MgCl_2$ were adjusted to 0.5 mM and KCl to 50 mM. Reactions were

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initiated by adding the enzyme to a final concentration of 10 nM, incubated for 10 min at 27 °C and stopped by adding equal volume of formamide/20 mM of EDTA. Products were analyzed on 15% acrylamide/urea sequencing gel. The RNA-independent UTP polymerization reactions contained enzyme and a 100 μM of UTP plus [α-^{32}P]UTP mixture (~3000 cpm/pmol). Kinetic parameters for UTP incorporation were determined in a filter-based TUTase assay with [α-^{32}P]UTP under the same conditions as above except that RNA concentration was maintained at 1 μM [13].

3. Results and discussion

3.1. Identification of potential uridylyltransferases

We searched the GeneDB databases (http://www.genedb.org) with RET1 and RET2 protein sequences and found four conserved predicted proteins in the *T. brucei* and *Leishmania major* genomes that display a high degree of similarity, e-value of 10^{-5} or below (Fig. 1). These predicted proteins with GeneDB IDs Tb10.6k15.3370, Tb11.01.7300, Tb11.02.5820 and Tb10.100.0050 were designated TbTUT3, TbTUT4, TbTUT5 and TbTUT6, respectively. An analysis of raw genomic contigs shows that the open reading frame for TbTUT5 (Tb11.02.5820) can be extended further upstream to include 86 more amino acids. Homologs of all these proteins have been found among the *L. major* predicted protein sequences deposited in GenBank: CAC18866 (LmTUT3), or in the GeneDB database: LmjF32.2450 (LmTUT4), LmjF28.0780 (LmTUT5) and L3640.07 (LmTUT6).

More distant homologs could be detected in human, mouse, *A. thaliana*, *Schizossacharomyces pombe* and other genomes when the NCBI GenBank database was searched with a profile (Position Specific Score Matrix) generated from trypanosomal RET1 and RET2 protein sequences (not shown). Most of the significant hits were predicted proteins of unknown function that clearly belong to the pol-β type nucleotidyltransferases. Searches with a profiles generated from trypanosomal poly(A) polymerases produced an entirely different set of proteins (not shown).

TbTUT3 (Tb10.6k15.3370) was chosen for experimental analysis because of the high degree of homology to RET1 TUTase (20% identity, 33% similarity). Also, TbTUT3 and LmTUT3 contain a C2H2 zinc finger motif located to the N-terminus of the catalytic domain. The deletion of zinc finger or removal of tightly bound zinc has been shown to be deleterious for LtRET1 activity [13]. The 99.3 kDa TbTUT3 protein contained a conserved nucleotidyltransferase motif and another zinc finger domain (GATA, positions 849–888), as predicted by SMART analysis (http://smart.embl-heidelberg.de/smart).

3.2. Expression and purification of TbTUT3

Attempts to produce the active trypanosomal protein in *E. coli* failed, but expression in *L. tarentolae* system [9] proved successful. The full-length TbTUT3 gene was amplified from *T. brucei* genomic DNA, sequenced and cloned into the *Bam*HI site of the pX [15] *Leishmania* expression vector with a fused C-terminal TAP tag [10]. This affinity tag consists of a calmodulin binding peptide and protein A and allows for efficient detection and two-step affinity purification of the fusion protein. Expression was monitored with the PAP reagent (Sigma) that recognizes the protein A moiety. TbTUT3-TAP fusion was expressed as soluble, non-membrane associated protein and was not detected in extracts from purified mitochondria (Fig. 2A). The mitochondrial extracts were tested for

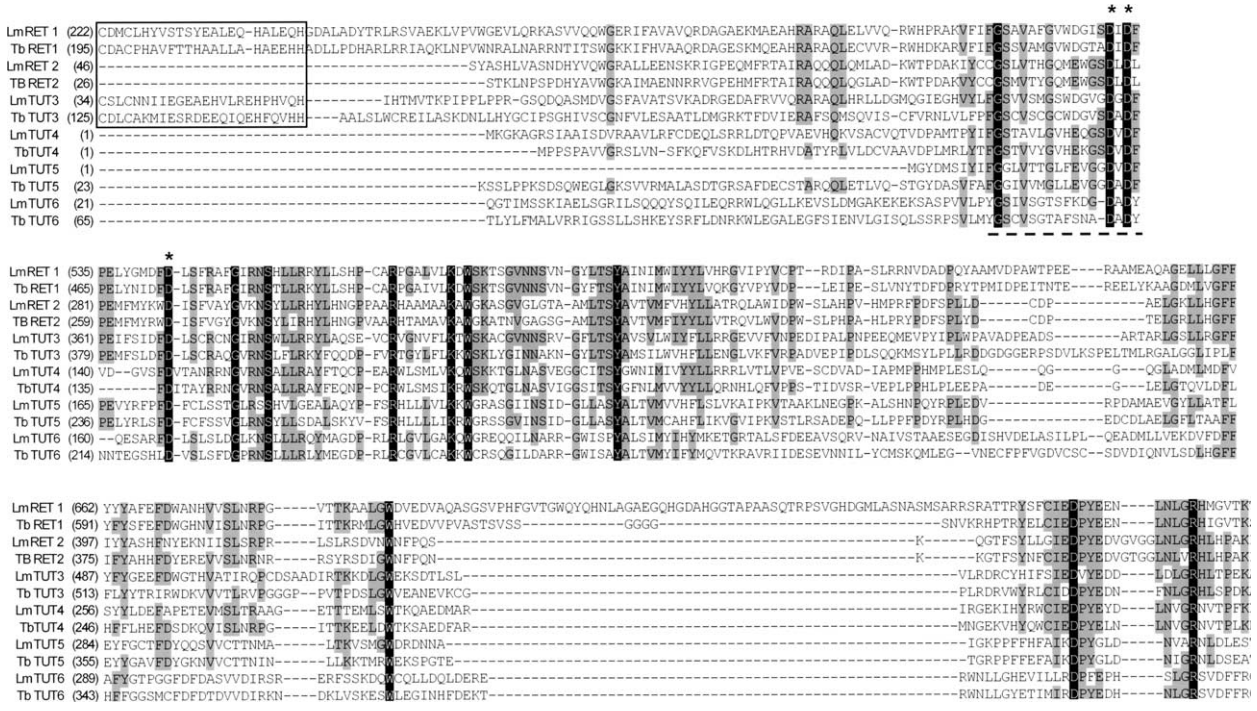

Fig. 1. Partial multiple alignment of trypanosomal TUTases. Protein sequences were identified by Blast searches of *L. major* and *T. brucei* GeneDB databases with RET1 and RET2 sequences. Alignment was performed with the ClustalW algorithm. Non-conserved regions were omitted. Identical amino acids are in white on black background, similar are black on grey background. The signature sequence of Pol β superfamily of nucleotidyltransferases is underlined. Asterisks indicate aspartate residues in RET1 that are essential for catalysis [13].

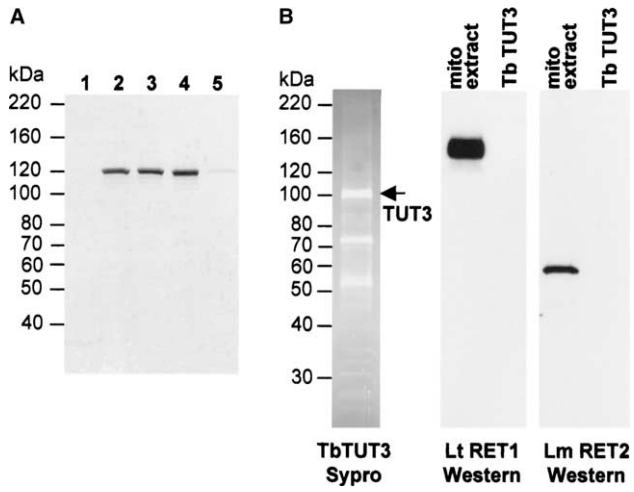

Fig. 2. TbTUT3 is a non-mitochondrial protein. (A) Expression of TbTUT3 in *L. tarentolae*. Proteins from 5×10^6 *L. tarentolae* cells were separated on 8–16% SDS gel, transferred to a nitrocellulose membrane and treated with PAP reagent to detect protein A fusions. 1, *L. tarentolae* cells; 2, *L. tarentolae* cells carrying pTAP-TbTUT3 plasmid; 3, cytoplasmic fraction; 4, S100 cytoplasmic fraction; 5, extract of purified mitochondria (20 μg of total protein). (B) Purification of TbTUT3. Approximately 0.1 μg of protein eluted from the calmodulin-agarose column was separated on an 8–16% SDS gel and stained with Sypro Ruby. Twice this amount was analyzed by Western blotting for the presence of RET1 or RET2 contamination. Polyclonal antibodies against recombinant *L. tarentoale* RET1 and *L. major* RET2 were utilized in this analysis. Mitochondrial extract (10 μg of total protein) was used as a positive control.

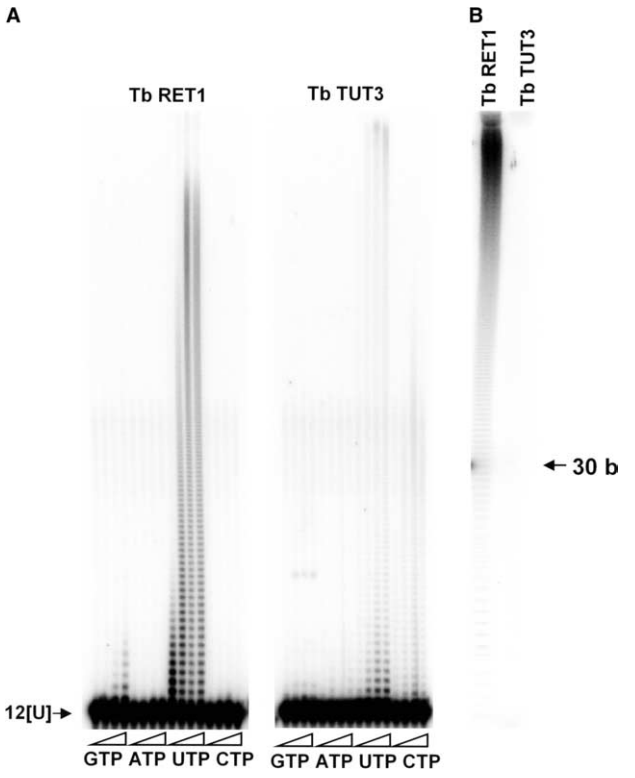

Fig. 3. TbTUT3 is a UTP-specific RNA nucleotidyltransferase. (A) RNA-dependent UTP polymerization. The reactions were performed with the 12[U] synthetic RNA [9] at 1, 10, 100 and 500 μM of the respective NTPs. Recombinant TbRET1 was analyzed in a parallel experiment as a reference. Products were separated on a 15% denaturing acrylamide/urea gel. (B) RNA-independent UTP polymerization by TbRET1 and TbTUT3. Arrow indicates a position of the 30-mer RNA.

the presence of RNA editing TUTases 1 and 2 to provide a positive control (Fig. 2B). This suggests a non-mitochondrial localization of TbTUT3, hence, a function unrelated to RNA editing. Clearly, the intracellular localization of TbTUT3 must be determined by further experiments.

Tandem affinity purification [16] of the TbTUT3 from total cell extract resulted in a preparation that contained a band of the expected size and two other bands (Fig. 2B). Based on our prior experience with this expression system, contaminating proteins are likely to be hsp70 and tubulin. We have previously used Leishmania system to express TbRET2 [9] and observed these proteins to co-purify with a TAP-tagged RET2 along with LC1 protein, which directly interacts with RET2 within core editing complex [17]. The presence of the HSP 70 may indicate partial misfolding of TbTUT3. The lack of RET1 and RET2 contamination in TbTUT3 preparation was confirmed by Western blot analysis (Fig. 2B).

3.3. TbTUT3 is an RNA uridylyltransferase

Recombinant TbTUT3 was enzymatically active as a 3′-terminal RNA uridylyltransferase. A high-resolution analysis of reaction products showed a processive incorporation pattern, similar to that of recombinant RET1 (Fig. 3A). Remarkably, the positioning of a C2H2 zinc finger motif with respect to the catalytic domain is conserved in LtRET1, TbRET1, TbTUT3 and LmTUT3 (Fig. 1). It is possible that the zinc finger is involved in the binding of the growing poly(U) product, thus conferring processivity to the polymerase reaction. RET2 lacks the zinc finger and adds mainly one UMP residue to a single-stranded RNA primer [9,11]. The precise role of the zinc finger remains to be determined.

There were, however, substantial differences in the enzymatic properties of TbRET1 and TbTUT3. The latter enzyme, unlike RET1 [13], does not polymerize UTP in the absence of RNA primer (Fig. 3B). This may reflect a different mode of the 3′-end RNA recognition: in RET1, UTP can interact with an RNA recognition site with sufficient affinity to serve as an acceptor for the incoming nucleotide triphosphate. Steady-state catalytic parameters were determined by a UTP polymerization assay based on RNA binding to DE81 filters [7]. The apparent K_m for UTP was very similar for the two enzymes, whereas the V_{max} for TbTUT3 was ~30-fold lower than that for RET1 (Table 1), which is consistent with the observed lower UTP incorporation efficiency of TbTUT3.

The requirements for di- and monovalent cations for optimal UTP polymerization activity also differed between RET1 and TbTUT3 (Fig. 4). The Mg^{2+} optima were 10 and 0.5 mM, respectively, and TbTUT3 TUTase activity was stimulated by KCl up to 50 mM and tolerated up to 200 mM salt whereas RET1 TUTase activity was severely inhibited at salt concentrations above 10 mM. Given the significant dissimilarity in

Table 1
Kinetic parameters of TbRET1 and TbTUT3 for UTP incorporation

Enzyme	K_m (mM)	V_{max} (μM/min)
TbRET1	18–28	1.7–2.3
TbTUT3	36–48	0.06–0.08

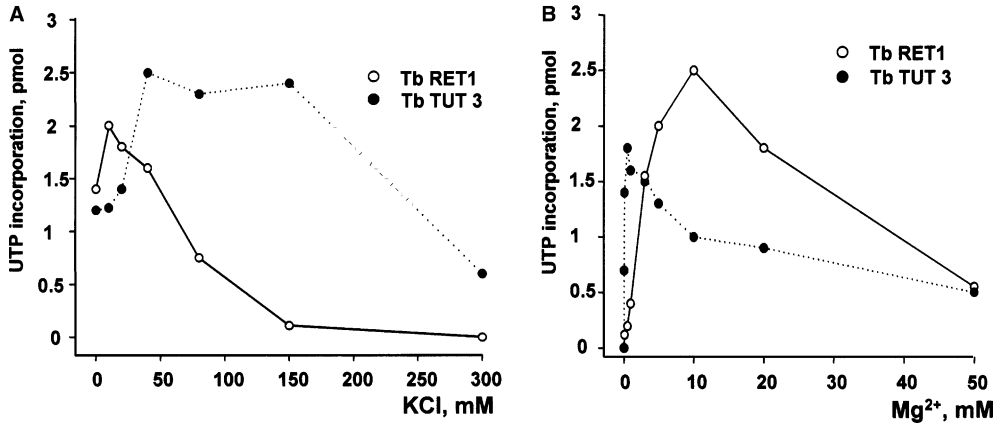

Fig. 4. TbTUT3 requirements for mono- and divalent ions. (A) Effect of potassium chloride concentration on UTP incorporation activity. (B) Effect of Mg^{2+} concentration on UTP incorporation by RET1 and TbTUT3.

enzymatic properties of RET1 and TUT3, it is plausible that in previous cell fractionations, the TUT3 activity may have not been detected under the conditions optimized for the dominant RET1 activity [5].

This paper presents the first experimental evidence that additional 3′ TUTases could be discovered in genomic databases by data mining with RET1 and RET2 protein sequences. The expression and characterization of the TbTUT3 was performed as a proof of principle, but further work must be performed to determine the sub-cellular localization and biological role of uridylyltransferases described in this work. Expanding the register of UTP-specific enzymes may enable prediction of conserved protein features that confer UTP specificity to these template-independent polymerases. The finding new TUTases in higher eukaryotes may increase our understanding of the role of these enzymes in RNA processing [2].

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